Differential ruminal degradation of alfalfa proteins

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Chen, D., Peel, M. D., Olson, K. C., Weimer, B. C. and DeWald, D. B. 2009. **Differential ruminal degradation of alfalfa proteins**. Can. J. Plant Sci. **89**: 1065–1074. Alfalfa (*Medicago sativa* L.) has high crude protein that is rapidly and extensively degraded in the rumen. Our objective was to develop a protocol where individual proteins could be characterized for their ruminal degradation. Proteins from individual genotypes of three alfalfa cultivars were characterized using fluorescence 2D difference gel electrophoresis combined with MALDI-TOF mass spectrometry for protein identification. Twenty-six proteins were characterized, representing between 33 and 41% of the total protein among genotypes. Variation for protein degradation was observed among proteins after 45 and 120 min of incubation in the rumen of a Holstein steer ($P<0.001$). After 45 min of ruminal incubation, nine proteins averaged 75% or more remaining, 12 had 50% or less remaining, and five were intermediate. After 120 min of ruminal incubation, four proteins averaged greater than 80%, seven between 80 and 50%, and 15 less than 50% remaining. Although all proteins were degraded over time, the rate and amount of degradation was dramatically different among them. The rate of digestion differed ($P=0.05$) for 3 and 10 proteins among genotypes after 45 and 120 min, respectively. Individual proteins characterized ranged in mass from 41 to 0.29% of the total mass of protein characterized. Total content of those proteins that differed for rate of digestion ranged from 7 to 1%. The results demonstrate that individual proteins can be characterized for their ruminal degradation. The ability to separate proteins based ruminal degradation suggests there is potential to select for protein that degrades more slowly and possibly escapes the rumen.

Key words: Alfalfa, protein, rumen, digestion

Chen, D., Peel, M. D., Olson, K. C., Weimer, B. C. et DeWald, D. B. 2009. **Dégradation des protéines de la luzerne dans le rumen**. Can. J. Plant Sci. **89**: 1065–1074. La luzerne (*Medicago sativa* L.) contient beaucoup de protéines brutes qui se dégradent vite et de façon importante dans le rumen. Les auteurs voulaient élaborer un protocole qui leur permettrait de caractériser les protéines selon leur dégradation dans le rumen. Dans ce but, ils ont caractérisé les protéines du génotype de trois cultivars de luzerne d'après les différences de fluorescence obtenues par électrophorèse bidimensionnelle couplée à la spectrométrie de masse MALDI-TOF pour l'identification des protéines. Vingt-six protéines ont ainsi été caractérisées et représentaient entre 33 et 41% de la totalité des protéines des génotypes. Une variation dans la dégradation des protéines a été observée au bout de 45 à 120 minutes d'incubation dans le rumen d'un bouvillon Holstein ($P<0,001$). Après 45 minutes, en moyenne 75% ou plus de la masse de neuf protéines n'était pas dégradée, 50% ou moins de la masse de 12 protéines n'était pas dégradée et cinq protéines se situaient entre les deux. Après 120 minutes, quatre protéines gardaient plus de 80% de leur masse, sept en gardait de 80 à 50%, et 15 avaient moins de 50% de leur masse intacte. Bien que toutes les protéines finissent par être dégradées, la rapidité et l'importance de la dégradation varie considérablement. Trois à dix protéines des génotypes présentaient un taux de digestion différent ($P=0,05$) après 45 et 120 minutes, respectivement. La masse des différentes protéines représentait 41% à 0,29% de la masse totale des protéines caractérisées. La concentration de protéines dont le taux de digestion varie s'établit entre 7 et 1%. Ces résultats montrent qu'on pourrait caractériser les protéines en fonction de leur dégradation dans le rumen. Le fait qu'on puisse distinguer les protéines selon leur dégradation dans le rumen laisse croire qu'on pourrait sélectionner des protéines qui se dégradent plus lentement et pourraient éventuellement échapper à l'action du rumen.

Mots clés: Luzerne, protéine, rumen, digestion

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Abbreviations: **2D-DIGE**, two-dimensional difference gel electrophoresis; **FRRL**, Forage and Range Research Lab; **MP**, metabolizable protein; **MS**, mass spectrometry

A major goal of forage production is to provide a feed with sufficient protein to meet the requirements of livestock, particularly dairy animals. The first step to reach this goal is to produce forage with adequate crude protein (crude protein is computed by measuring the nitrogen percentage in a dried sample and multiplying by 6.25). However, ruminant animals' needs are actually met by metabolizable protein [National Research Council (NRC) 1996, 2001]. Metabolizable protein (MP) is the combination of feed protein (actual protein not N) that escapes degradation in the rumen plus protein synthesized by ruminal microorganisms. The efficiency with which the forage crude protein is utilized as MP by ruminant livestock depends partly on its ruminal degradability (NRC 1996, 2001).

Alfalfa (*Medicago sativa* L.) has one of the highest crude protein contents among forage crops, but it is rapidly and extensively degraded by rumen microorganisms. Synthesized microbial protein is subsequently used by the ruminal host as a source of amino acids for the production of animal protein. However, when the animal's protein requirements are high, microbial protein is insufficient to meet its nutritional needs. Furthermore, if protein is degraded too quickly in the rumen, more ammonia may be produced than can be used by the microbial population leading to inefficient conversion of feed N to microbial protein and excretion of excess ammonia as urea. Thus, alfalfa protein utilization could be improved by increasing the proportion of the protein that avoids degradation in the rumen and decreasing the degradation rate of ruminally degradable protein. This alteration would increase MP that passes into the small intestine where it can be used directly by the animal. Additionally, increasing the amount of protein resistant to rapid microbial degradation may reduce the bloat danger associated with grazing alfalfa.

Previous studies measuring ruminal crude protein degradability indicate that extending intact protein in the first 2 h provides a significant benefit to the animal (Nugent and Mangan 1981). Condensed tannins are effective inhibitors of rumen microorganisms and may contribute to reduced rumen protein degradation and increased bypass protein. Condensed tannins are found in the nonbloating legumes birdsfoot trefoil (*Lotus corniculatus* L.) and sainfoin (*Onobrychis vicifolia* Scop.) as well as others, but are not present in alfalfa (Howarth 1988). Herbage proteins have been characterized as insoluble and soluble. The insoluble proteins are those typically associated with cell walls and membranes (Lyttleton 1973). More recently CP in forage has been described in three sub-fractions – A, B1, B2, and B3 – and an unavailable fraction, C, where each refers to that fraction of CP pattern of solubility and degradability in the rumen (Elizalde et al. 1999). Solubility, protein type, and conformation are reported to govern protein degradation in the rumen, but even this has proved to be an imperfect correlation (Mahadevan et al. 1987). Beyond this, little is known about the actual proteins that degrade rapidly in the rumen.

Improvement of alfalfa with increased protein bypass will require not only variation for the trait but the ability to consistently separate genotypes for their rate of protein digestion. Previous research has demonstrated variability among alfalfa germplasm for ruminal degradation of total crude protein (Broderick and Buxton 1991; Skinner et al. 1994; Rooney et al. 1997; Tremblay et al. 2000, 2003). Skinner et al. (1994) suggest that cultivars could be developed with decreased protein degradability if selection was done on individual plants. This is supported by Tremblay et al. (2003) who found variation for ruminal undegradable proteins among genotypes within cultivars, but not among cultivars. Rooney et al. (1997) stated: "If selection for reduced protein degradability is to be successful, in vitro methods of estimating ruminal protein degradability are critical due to the large number of samples that must be evaluated."

Currently, there are no known reports that describe the ruminal degradation of specific alfalfa proteins. Making gains in a breeding program is limited without the ability to differentiate between genotypes; this is especially true when there is not an easily defined phenotype. The ability to identify and select for specific proteins with reduced degradation would enable the success of a selection program with the objective of improving the feed value of alfalfa protein.

Increasing protein escape from the rumen is a global issue and would provide an economic benefit to the livestock industry, particularly the dairy industry. Our objective was to develop a protocol whereby major individual proteins could be identified and the ruminal degradation of those individual proteins could be quantified after 2 h of digestion in the rumen.

MATERIALS AND METHODS

Plant Growth

Materials tested originated from three sources of alfalfa (*Medicago sativa* spp.) germplasm that included Vernal, BC79, and SaltII. Vernal (*M. sativa* ssp. *sativa*) (Graber 1956) is commonly used as a check cultivar in studies involving alfalfa throughout the United States of America, where semi-dormant alfalfa is produced. BC79 and SaltII are breeding populations developed at the USDA-ARS Forage and Range Research Lab (FRRL) (Logan, UT). BC79 is derived primarily from a *M. sativa* ssp. *falcata* background. SaltII is a population derived from a *M. sativa* ssp. *sativa* background that was selected for salt tolerance following Peel et al. (2004). BC79 and SaltII were chosen because of their distinctly different genetic backgrounds to maximize the likelihood of genetic differences.

One genotype consisting of three clonally replicated plants from each germplasm source (Vernal, BC79 and SaltII) was utilized. These plants were grown in a

greenhouse in a randomized complete block design with each replication represented by a clone from each genotype. Plants were cut to 5 cm on 2003 Dec. 22 to initiate new growth. Growth was encouraged by supplying the plants with a supplemented (150 μmol m^{-2} s^{-1} 1.8 m below the light source) 18 h photoperiod. Plants were harvested during the first week of 2004 February at 10% bloom. The genotypes utilized were selected from a larger group within each germplasm source based on their similarity in maturity at harvest. Prior to harvest all plants were rated for percent flowering based on the number of flowers per stem for each plant to obtain relative maturity and select genotypes for protein characterization. This was done to maximize the likelihood that differences were genetic and not due to a difference in growth stage or from harvesting at a different time. Since alfalfa is open pollinated and populations are highly heterogeneous, a single genotype (only clones of the same original plant) from each germplasm source were used to increase the likelihood of detecting variation for protein degradation as suggested by Skinner et al. (1994). This approach and the single genotype from each germplasm source precludes us from using the results to make statements about the genetic variation among germplasm sources since there is no population structure, but increased the likelihood of detecting differences among the sampled genotypes. This was not a limitation to achieve our objective, which was to develop a protocol to detect degradation differences among and between alfalfa proteins. Harvested plants, three from each genotype, were dried at 32°C to simulate field conditions. Dried forage from each plant was ground to pass through a 2-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ). Samples from the three clonally replicated plants were maintained separate and represented replications in the rumen fermentation and subsequent protein extraction and characterization.

Ruminal Protein Degradation

All incubations were performed in the rumen of a ruminally cannulated Holstein steer that was consuming a mixed grass and alfalfa diet. Samples of 1.25 g of each alfalfa substrate were sealed in 5 × 10 cm Dacron bags (Ankom, Fairport, NY) with a 50 ± 15 μm pore size, and heat-sealed using an impulse sealer (model MP-8; Midwest Pacific from Ankom, Fairport, NY). Samples for each time point were confined in 36 × 42 cm polyester mesh bags to ensure similar location within the rumen and to assist in retrieval. Nine Dacron bags of each of the three genotypes three from each plant were filled. This represented the three replicates of each of three ruminal incubation times. One set of replicate bags was incubated on each of three different days. Incubation times were 0, 45, and 120 min. Bags were placed in the rumen in reverse order (i.e., the 120 min bags were inserted first) and all bags were removed simultaneously at 0 min. Bags from time 0 were not placed in the rumen, but were subjected to the same rinse procedure as the incubated bags. All bags from a replicate were rinsed together to remove contamination with ruminal feed contents in a Kenmore heavy-duty, top-loading washing machine (Sears, Roebuck, and Co., Chicago, IL) for 10 rinse cycles using room-temperature tap water. Each rinse cycle consisted of a 1-min agitation and a 2-min spin. Bags with residues were dried overnight at 40°C, opened and the residue was reground in a coffee mill before protein analysis.

Protein Extraction, Labeling and 2D-DIGE

Total protein was extracted from the alfalfa residues as described by Giavalisco et al. (2003). The samples were ground in liquid N_2 to a fine powder using a mortar and pestle. Lysis buffer (0.8 mL: 7 M urea, 2 M thiourea, 4% CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid), 0.8% IPG (immobilized pH gradient); Amersham Biosciences Inc./GE Healthcare Life Sciences, Pittsburgh, PA) containing protease inhibitors (Complete™, Molecular Biochemicals, Roche, Indianapolis, IN) was mixed with 200 mg of the alfalfa powder of each sample and the samples were transferred to a 1.5-mL microfuge tube. This slurry was mixed briefly using a vortex and kept in the dark on ice for 10 min. Subsequently, the samples were centrifuged at 10 000 rpm at 4°C for 15 min and the supernatants were collected. The extracts were prepared for electrophoresis using the Bio-Rad ReadyPrep™ (Bio-Rad Laboratories, Hercules, CA) two-dimensional difference gel electrophoresis (2D DIGE) Cleanup Kit. A 2D DIGE system – Ettan™ DIGE (Amersham Biosciences) was used to separate the proteins in each sample.

Protein labeling for 2D DIGE was performed as described in the Amersham Biosciences protocol (Amersham Biosciences 2002). The 2D DIGE chemistry relied on N-hydroxysuccinimide ester reagents for low-stoichiometry labeling of e-amino groups on the lysine side chains. Labeling reactions were done according to Amersham's protocols so that ~2 to 5% of the total proteins were labeled. This biased the reactions so that quantification was performed on protein molecules that had only been labeled once.

Rumen-exposed and control protein samples (50 μg) were labeled using 400 pmol of Cy2, Cy3 or Cy5 dye for the 0, 45, and 120 min degradation points, respectively. For labeling, the dyes were added to the samples and kept on ice for 30 min in the dark. Subsequently, the 2D DIGE analyses were done as described by the manufacturer. Briefly, a lysine solution (1 μL of a 10 mM) was added to each of the samples to stop the reactions. Samples were then maintained on ice in the dark for an additional 10 min. Following the dye labeling reactions, equal volumes of the individually fluorescently labeled protein mixtures were combined.

An equal volume of the 2 × sample buffer [8M urea, 4% CHAPS, 2% DTT (dithiothreitol) and 2% Pharmalyte] was added to the individually fluorescently labeled

protein solutions to bring the final sample volume to 185 μL. The entire volume was placed onto a Bio-Rad IPG strip (11 cm, pH 3–10) for overnight hydration at 21°C. Isoelectric focusing was carried out using a Bio-Rad PROTEAN IEF cell with 50 μA per gel and a total of 25 000 volt-hours. The second dimension separation was performed using 11 cm × 15 cm, 10–20% acrylamide slab gels run on a BioRad Criterion Cell at constant voltage (200 V) and a running buffer of 25 mM tri-base, 190 mM glycine and 0.1% SDS.

2D-DIGE Image Scanning and Analysis

The 2D-DIGE images were obtained using a Typhoon 9400 scanner (GE Healthcare Bio-Sciences, Piscataway, NJ) operating in fluorescence mode. Images of the fluorescently labeled proteins in gels were captured using an excitation wavelength of 488 nm and emission wavelength of 520 nm for Cy2-labeled, an excitation wavelength of 532 nm and an emission wavelength of 580 nm for Cy3-labeled, and an excitation wavelength of 633 nm and an emission wavelength of 670 nm for Cy5-labeled proteins. Phoretix 2D Evolution software (Nonlinear Dynamics Inc, Durham, NC) was used to analyze the DIGE images.

Protein Identification by Mass Spectrometry

The 2D-DIGE gels were imaged and 26 specific protein spots were robotically excised as gel plugs using an Etten Spot Picker (Amersham Biosciences, Inc.; Piscataway, NJ). Protein spots were selected with medium to strong spot intensity to ensure sufficient protein was present for detection with the mass spectrometer. The collected proteins were digested in situ with trypsin, as described by Jimenez (1998). The resultant peptides were characterized using a nano-LC-MS-MS on a Q-Tof Primer tandem mass spectrometer (Waters, Manchester, UK). The peptide samples (2 μL) were introduced into a NanoACQUITY Sample Manager (Waters, Manchester, UK) for analysis with a Symmetry® C_{18} trapping column (180 μM × 20 mm) at 5 μL min^{-1}. The peptides were eluted from a 75 μm × 10 cm Atlantis™ dC_{18} column with a 65 min gradient (3% B for 1 min, 3–35% B over 30 min, 35% B for 1 min, 35–90% B over 2 min, 90% B for 4 min, 90–3% B over 1 min and 3% B for 26 min) at 300 nL min^{-1} using an NanoACQUITY UPLC (Waters, Manchester, UK). For this system, solvent A was composed of 99.9% water and 0.1% formic acid. Solvent B was composed of 90.9% acetonitrile, 9% water and 0.1% formic acid. The mass spectrometry (MS) analysis and product ion MS/MS scan times were 1.0 and 1.9 s, respectively. The collision offset was automatically determined based on precursor mass and ion charge state. Peptide product ion data were searched against the NCBI non-redundant protein database using the Mascot search engine (Matrix Science, London, UK, http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS 6/17/09). The search results with at least two peptide matches having a significant Probability Based Mowse Score are presented in the results.

Data Analysis

The quantities of each individual protein in control and rumen-digested samples were calculated based on pixel intensity on the 2D-DIGE gel images as described. Percentage of pixels remaining at each incubation time was the primary unit reported. The data were analyzed as a randomized complete block design using the PROC-ANOVA procedure of SAS (SAS Institute, Inc., Cary, NC). Replicate bags were the experimental unit and were designated as random effects. Alfalfa genotype and each identified protein were considered fixed effects. Mean separations of proteins within a genotype were made on the basis of the Fisher's protected least significant difference (LSD) test at the 0.05 level of probability. To make comparisons of individual proteins between genotypes, data from an individual protein across genotypes was analyzed independently. When differences were detected a t-test was used to show those differences (Steel and Torrie 1980).

RESULTS AND DISCUSSION

Electrophoretic Protein Analysis

Individual proteins that altogether compose the proteome of a particular plant or tissue can be separated on two-dimensional electrophoretic gels, providing a snapshot of the numbers and relative quantity of resolved proteins. High-resolution 2D gel electrophoretic systems are widely used in proteomics studies of multiple biological systems. However, gel-to-gel variability means that the protein expression patterns on an individual 2D gel can be challenging to repeat and it is often difficult to identify changes in protein quantity (Zhou 2002). For this study we chose to use the 2D-DIGE technique developed by Minden and colleagues (Unlu 1997), which not only separates proteins with a broad range of isoelectric points (3–10) and molecular weights (10 kDa to 200 kDa) (Fig. 1), but also improves the reproducibility of analyzing protein expression, since the control and treatment samples are run on the same gel.

To determine the extent our rinsing protocol caused protein loss, we first examined the total number of alfalfa proteins that could be separated on the 2D gel system by analyzing the unrinsed undigested control sample (Fig. 1a) that had not been subjected to rumen digestion or rinsing. Gels from one BC79 replication are shown in Fig. 1 for illustration. The gel of this sample displayed approximately 500 protein spots, with proteins ranging from greater than 100 kDa to less than 10 kDa in molecular mass across the entire pI (3–10) range. A preliminary comparison was made of the amount of actual proteins measured from these unrinsed samples (Fig. 1a) and the residues that were rinsed but not subjected to digestion (Fig. 1b). It was noted from this comparison that the amount of actual protein

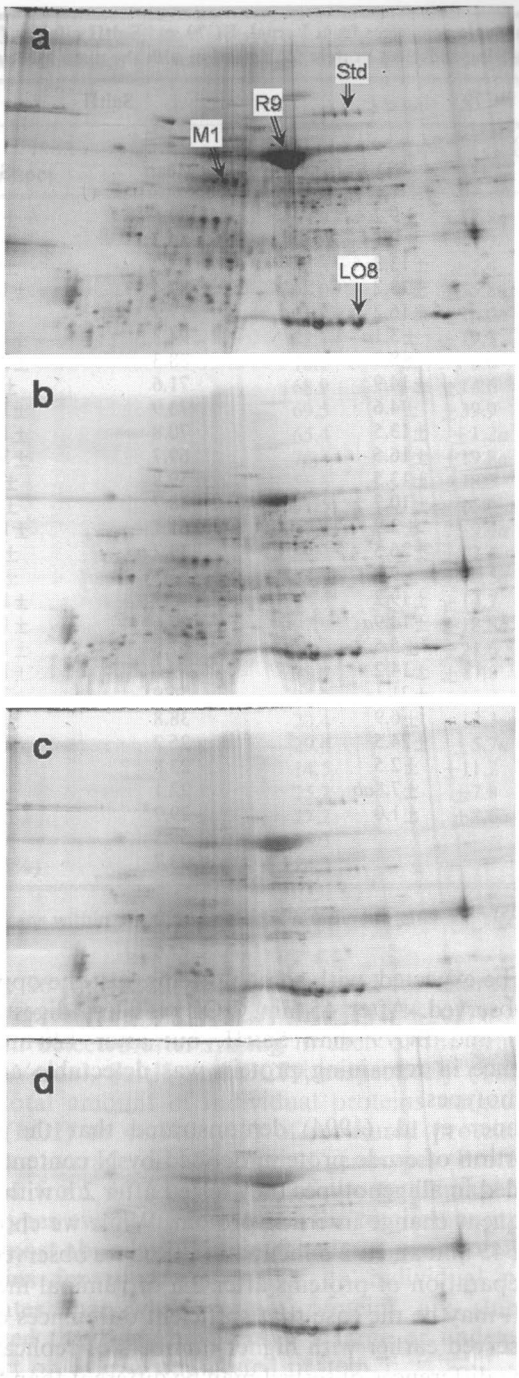

Fig. 1. Differential degradation of proteins observed using two-dimensional gel electrophoresis; gels are from one replication of the BC79 samples with three protein spots and the internal standard labeled. a, Unfermented and unrinsed forage sample; b, time zero rinsed and unfermented; c, following 45 min of fermentation; d, following 120 min of fermentation. Gels show the protein content before and after rumen degradation for 45 and 120 min. Gel b shows approximately 500 spots in pI range of 3–10 and mol wt. range of 10 to 200 kDa.

changed little after rinsing (data not reported). This was expected because the N that disappears during the rinsing of unfermented samples is generally considered to be non-protein N (Broderick 1994), and would not have contributed to the individual proteins extracted from the samples and separated on the gels.

We then analyzed protein samples from different degradation time points (0, 45, 120 min) that were labeled with different fluorescence dyes (Cy2, Cy3 and Cy5) and separated on a single 2D gel. The gel was scanned to obtain Cy2, Cy3 or Cy5 fluorescence representing the quantity of individual proteins in spots from different time points (Fig. 1b, c and d). A dramatic reduction in quantities of many proteins were observed between the rinsed undigested (Fig. 1b) and samples that were rumen-exposed for 45 min (Fig. 1c), and these reductions were more pronounced after 120 min (Fig. 1d). These same trends were observed for the other genotypes, so we proceeded with the quantification of total and individual protein degradation in the rumen treated samples.

Quantification of Protein Degradation

In the overall analysis significant variation was detected between genotypes, time of ruminal incubation and proteins (Table 1). Furthermore, an analysis within incubation time revealed significant variation for individual proteins ($P<0.001$) at both 0–45 and 0–120 min. Significant variation for protein degradation was also observed for genotype at both 0–45 ($P=0.004$) and 0–120 ($P=0.003$) min. Even though most proteins were uniformly digested across genotypes, analysis of individual proteins across genotypes demonstrated that some differed ($P<0.05$) for rate of digestion (Tables 2 and 3).

After 45 min of digestion, nine proteins averaged 75% or more of their mass remaining, 12 had 50% or less remaining, and five were intermediate (Table 2). The percent of protein remaining in the residue after 45 min of ruminal incubation differed among genotypes for proteins LO2, M1, and R8. The proteins LO2 and M1 tended to be more highly digested proteins, while R8 was more stable across genotypes. After 120 min of digestion four proteins averaged greater than 80% mass

Table 1. Significant levels from the analysis of variance for percent of individual alfalfa proteins remaining after 45 and 120 min of in situ ruminal incubation

	Significance level		
Source	Combined analysis	45 min	120 min.
Genotype (G)	<0.001	0.004	0.003
Incubation time (T)	<0.001	–	–
Protein (P)	<0.001	<0.001	<0.001
G × P	0.003	0.19	0.29
G × T	0.422	–	–

Table 2. Percent individual proteins remaining after 45 min of ruminal fermentation in single genotypes from Vernal, BC79 and SaltII alfalfas. The 26 proteins were chosen for characterization based on medium to strong spot intensity ensuring sufficient protein for detection with the mass spectrometer

	Vernal		BC79		SaltII	
	(%)					
Protein spot	Mean	SD	Mean	SD	Mean	SD
R9	96.2	±13.9	87.9	±9.2	89.6	±9.9
LO9	92.0	±3.5	87.7	±8.5	86.3	±8.7
LO1	78.3	±11.6	94.1	±13.3	91.2	±3.1
R1	88.1	±5.6	81.2	±13.2	87.4	±19.8
LO8	93.0	±10.8	82.3	±16.2	77.9	±8.0
R8	88.4	±3.8*ab*	70.1	±3.1*a*	94.3	±16.2*b*
R2	90.3	±8.9	73.2	±8.2	88.3	±22.8
LO5	86.9	±8.5	82.5	±14.9	71.6	±9.8
LO7	79.1	±15.8	74.7	±4.6	73.9	±15.9
R3	74.5	±6.4	69.6	±13.5	70.8	±10.8
LO4	73.3	±22.9	60.8	±16.5	69.7	±30.0
U3	72.0	±21.6	68.6	±13.5	59.8	±6.8
R7	70.0	±12.8	69.9	±10.7	48.7	±5.9
L1	68.7	±13.9	58.4	±2.9	61.1	±14.5
L2	64.0	±16.2	44.1	±26.5	39.7	±9.0
R4	58.3	±20.9	34.3	±9.3	53.3	±9.7
U1	49.4	±9.6	44.4	±19.2	48.7	±11.1
LO2	59.0	±29.0*a*	55.6	±14.9*a*	27.6	±10.0*b*
U4	39.4	±11.5	38.7	±5.5	61.8	±18.0
R6	49.1	±11.5	39.4	±14.2	49.0	±17.8
U2	41.5	±11.2	34.8	±21.1	56.9	±22.5
R5	55.3	±12.4	37.5	±6.9	38.8	±8.7
LO3	27.4	±16.3	54.2	±24.5	25.2	±20.9
M2	41.3	±15.3	35.7	±2.5	23.8	±8.8
M1	43.1	±5.9*a*	31.7	±7.8*ab*	22.1	±8.8*b*
LO6	33.6	±3.3	26.8	±1.0	29.7	±9.4
Mean	65.9		59.2		59.5	
LSD (5%)	21.2		21.8		23.7	

a, *b* Means within a row followed by a different letter are significantly different (0.05).

remaining, seven between 80 and 50%, and 15 less than 50% remaining (Table 3). The amount of protein remaining in the residue after 120 min of incubation differed among genotypes for proteins LO5, LO8, M1, M2, R2, R3, R6, R7, R8 and U4.

Several proteins were stable across the three genotypes such as R9, which had 96.2, 87.9, and 89.6% protein remaining after 45 min digestion for Vernal, BC79, and SaltII, respectively. Protein LO5 was relatively stable after 45 min, but was nearly completely digested in BC79 and SaltII after 120 min. The opposite was observed for LO6, which averaged about 30% remaining after 45 min with about 20% remaining after 120 min for a change of 10% compared with the average change of 15% for all other proteins.

A few proteins, such as LO9, were resistant to digestion, even after 120 min, but, generally, proteins were uniformly more digested after 120 min compared with 45 min and differences among proteins became more pronounced. After 45 min, no difference in mass reduction was observed between the three genotypes for the protein LO5; however, after 120 min of incubation, the remaining amount of this protein was different in each genotype with very little change observed in Vernal to only 34% remaining in SaltII (Table 2 and 3). As might be expected, with some proteins, just the opposite was observed. After 45 min, LO2 was less digested in Vernal and BC79 than SaltII, but after 120 min no difference in remaining protein was detectable among the genotypes.

Skinner et al. (1994) demonstrated that the same proportion of crude protein, defined by N content, was degraded in all genotypes they tested after 2 h with little subsequent change in crude protein. While we chose to look a 45 min and a 2 h incubation time we observed the best separation of proteins after 2 h of ruminal incubation. It may be the case that sufficient differences could be observed earlier with higher numbers of replications and the differences observed may be different than those observed after 2 h. It is important to note that the three genotypes we characterized are by no means a representation of the genetic variation found within alfalfa, rather a sample specifically chosen to determine if individual proteins could be separated based on their ruminal digestion. Now that the protocol has been developed, characterization of a larger number of genotypes likely would reveal genotypic differences similar to observations by Tremblay et al. (2003) but on an individual protein basis.

Table 3. Percent of individual proteins remaining after 120 min of ruminal fermentation in single genotypes from Vernal, BC79 and SaltII alfalfas. The 26 proteins were chosen for characterization based on medium to strong spot intensity ensuring sufficient protein for detection with the mass spectrometer

	Vernal		BC79		SaltII	
	(%)					
Protein spot	Mean	SD	Mean	SD	Mean	SD
LO8	103.9	±12.8*a*	89.9	±1.2*ab*	77.2	±8.0*b*
LO9	96.8	±1.7	84.3	±9.1	89.7	±10.6
R9	86.3	±7.4	84.1	±3.7	82.4	±15.2
R2	96.0	±15.8*a*	62.8	±14.5*b*	90.6	±1.0*a*
R8	91.8	±16.0*a*	59.7	±10.0*b*	84.7	±10.9*ab*
LO1	72.1	±19.3	78.5	±10.2	76.0	±19.8
R1	78.1	±6.6	70.6	±12.2	72.6	±26.8
LO7	68.9	±14.6	58.2	±22.1	61.0	±3.1
LO4	69.5	±39.9	42.9	±12.4	52.6	±24.4
R3	65.4	±1.2*a*	48.5	±6.3*b*	50.1	±9.2*b*
LO5	75.4	±19.8*a*	47.6	±16.2*b*	34.1	±14.0*c*
L1	56.6	±18.3	48.5	±5.1	44.5	±15.5
U3	51.5	±15.0	46.0	±3.7	36.9	±12.1
U4	37.9	±9.4*a*	30.8	±3.7*a*	49.2	±6.9*b*
L2	39.9	±12.4	40.7	±6.5	29.9	±15.4
R7	55.1	±16.5*a*	33.6	±13.8*b*	16.5	±6.4*ab*
LO2	33.6	±14.7	31.6	±9.6	22.9	±15.1
R6	37.9	±18.8*a*	16.8	±16.1*ab*	29.4	±18.5*b*
R4	39.0	±21.6	19.9	±7.0	20.1	±8.3
R5	38.9	±17.8	21.0	±5.3	17.1	±2.8
U1	25.2	±13.3	28.0	±12.8	19.5	±15.0
LO3	20.4	±13.3	32.0	±15.2	12.4	±6.5
M1	29.4	±5.7*a*	21.5	±5.2*ab*	11.4	±8.3*b*
U2	14.5	±11.2	23.9	±14.7	22.6	±17.8
LO6	25.2	±7.8	15.1	±4.6	20.2	±2.2
M2	27.2	±8.6*a*	18.8	±5.7*ab*	12.3	±3.5*b*
Mean	55.3		44.4		43.7	
LSD (5%)	24.3		16.9		20.4	

a, *b* Means within a row followed by a different letter are significantly different (0.05).

The 26 individual proteins selected for characterization represented 40.7, 32.9, and 34.8% of the total protein detected after rinsing in each of the genotypes from Vernal, SaltII and BC79, respectively (Table 4). The total amount of individual proteins ranged from 0.21 (LO1) to 46.7% (R9) of measured protein mass with an average of 3.7% across genotypes. Protein R9 ranged from 46.7 to 36.2% of the total mass across genotypes averaging four times the total amount of the next most abundant protein. The large variation among proteins for total amount of an individual protein indicates that while certain proteins are stable or digested they may have either a large or undetectable impact on overall undigested protein.

An evaluation of the individual proteins that differed after 45 min of ruminal incubation revealed that proteins LO2 and R8 both contained less than 1% of the total protein characterized and protein M1 was 2.9, 5.1, and 3.8% of the protein characterized for Vernal, SaltII and BC79, respectively (Table 4). The same comparison of individual protein amounts after 120 min reveals that 5 of the 10 proteins that differed among genotypes contained 1% or less of the total protein mass characterized. The remaining four proteins ranged from 6.6% for protein R3 to 1% for protein LO5 in Vernal. It appears that of the proteins that differed for rate of digestion, proteins R3, LO8, M1 and M2, would represent viable options for significantly decreasing the amount of protein digested in the rumen. It should be noted that R9 by far constituted the largest amount of any individual protein and would be an obvious choice for modification. We did not observe significant differences between genotypes for digestion of protein R9, which is not surprising, given our small sample of genotypes. The genotypic sample we tested only allowed us to say that differences in ruminal degradation of protein can be measured and should not be misconstrued as a representation of variation among the populations or cultivar that they came from. Having stated this, it would not be unreasonable to expect that variation for ruminal degradation of protein R9 could be identified.

Protein Identification

Twenty-six spots in the 2D gels were selected for protein identification. Of the 26 proteins characterized for their rate of ruminal digestion 19 were identified using MS/MS (Table 5). Several of the proteins identified can be grouped by their implied chloroplast function. Included among this group are the photosystem proteins,

Table 4. Mean normalized pixel intensity prior to ruminal incubation for 26 selected proteins in single genotypes from Vernal, BC79 and SaltII alfalfas

	Vernal		SaltII		BC79	
Protein spot	Pixel #	% of selected	Pixel #	% of selected	Pixel #	% of selected
R9	3787.6	46.74	1838.2	36.24	1871.1	39.80
R1	897.6	11.08	309.5	6.10	431.9	9.19
LO9	763.6	9.42	452.6	8.92	323.1	6.87
R3	537.9	6.64	131.4	2.59	230.3	4.90
LO8	347.4	4.29	268.7	5.30	222.6	4.74
M1	232.9	2.87	258.6	5.10	177.3	3.77
M2	208.1	2.57	291.8	5.75	190.2	4.05
U2	169.7	2.09	82.1	1.62	79.7	1.69
U3	132.4	1.63	195.3	3.85	87.3	1.86
U1	116.0	1.43	156.8	3.09	62.0	1.32
Internal standard	100.0	1.23	100.0	1.97	100.0	2.13
LO5	80.1	0.99	135.8	2.68	71.4	1.52
R4	72.7	0.90	64.8	1.28	68.3	1.45
L1	71.8	0.89	128.3	2.53	108.0	2.30
L2	70.0	0.86	116.4	2.29	92.9	1.98
U4	68.1	0.84	68.9	1.36	43.5	0.93
LO3	63.9	0.79	58.5	1.15	50.1	1.07
R2	62.3	0.77	44.9	0.89	73.1	1.55
LO6	59.8	0.74	70.3	1.39	63.6	1.35
R5	49.4	0.61	58.9	1.16	57.0	1.21
LO4	42.0	0.52	63.0	1.24	36.2	0.77
R7	42.0	0.52	47.9	0.94	30.9	0.66
LO2	35.0	0.43	48.4	0.95	36.8	0.78
LO7	30.2	0.37	18.4	0.36	109.2	2.32
R6	25.5	0.32	22.6	0.45	52.5	1.12
R8	21.3	0.26	16.1	0.32	13.3	0.28
LO1	16.7	0.21	24.4	0.48	18.7	0.40
Total selected spot intensity	8104.1	100.0	5072.7	100.0	4701.0	100.0
Total spot intensity	19890.8		15441.5		13511.8	
Percent selected spot intensity	40.7%		32.9%		34.8%	

RuBisCo heavy (R9) and light subunits (U1 and U2), and the oxygen evolving enhancer protein (M1 and M2). The heavy subunit of RuBisCo (R9) is of particular interest because it constitutes such a large percentage of the total protein as previously discussed (Table 4). However, the overall goal of the effort to identify proteins was not to correlate degradation with functional groupings. The goal was to provide a list of known proteins, instead of arbitrarily numbered protein spots, that degrade differently and could serve as selectable markers for alfalfa improvement.

CONCLUSIONS

We successfully separated and characterized 26 major proteins from alfalfa samples that were degraded in the rumen of a fistulated steer. Additionally, we quantified the mass reduction of these proteins over a 120-min fermentation. It was observed that these proteins ranged from relatively stable to highly labile and differences in disappearance between genotypes for the same protein can be detected. Finally, through mass spectroscopy and protein data base searches, we were able to identify 19 of the 26 proteins characterized for ruminal degradation.

Our results suggest positive implications for plant breeding programs to reduce protein degradation in alfalfa, and likely other forages. Specifically, variability in amount and degradation among proteins can be determined and as such selection programs to modify the ratios of these proteins to increase the amount that degrades slowly may be possible. Further, the ability to identify the major proteins involved may prove of value as protein biomarkers in a selection program focused on rumen-stable proteins.

It appears from these results that the opportunity exists to improve the value of alfalfa as a dietary protein source by development of an alfalfa with more proteins that degrade slowly in the rumen and possibly more protein that escapes degradation in the rumen. These improvements would lead to more metabolizable protein for use by the animal, less nutritional disease (i.e., bloat), and less N contamination from animal waste in the environment.

Further research is needed to determine if these potential benefits are achievable. First, streamlined methodology will be needed to quickly, inexpensively, and quantitatively screen large numbers of genotypes for protein degradation biomarkers. Certain proteins would be likely candidates for biomarkers, such as the large subunit of RuBisCo (R9), which is much more abundant that any other protein. Second, the affect of a

Table 5. Identification using mass spectrometer of 19 proteins from alfalfa characterized for ruminal degradation

Spot ID[z]	Identified proteins	NCBI accession number	Peptides matched by MS/MS n	Sequence coverage %	Probability-based MOWSE Score n	Theoretical molecular weight Da	Observed molecular weight in gel Da	Theoretical pI	Observed pI
L1	Putative PSII P protein (*Trifolium pratense*)	BAE71271	3	13	124	28237	23000	7.12	5.6
L2	Putative PSII P protein (*Trifolium pratense*)	BAE71271	2	8	106	28237	23000	7.12	5.9
LO4	Chloroplast thioredoxin M-type	ABC46707	4	16	121	19828	15000	9.07	4.8
LO5	Pprg2 protein (*Medicago sativa*)	CAC86467	2	18	142	16582	18000	5.8	5.6
LO7	RNA binding protein	AAA33039	2	7	108	31944	27000	4.74	4.6
LO8	Ribulose bisphosphate carboxylase small chain	O65194	4	20	87	20238	16000	8.86	7.3
LO9	Ribulose bisphosphate carboxylase small chain	O65194	5	0	170	20238	16000	8.86	6.8
M1	Oxygen-evolving enhancer protein 1	CAA33408	4	8	82	35367	30000	5.84	5.4
M2	Oxygen-evolving enhancer protein 1	CAA33408	2	6	73	34719	30,000	8.73	5.6
R2	RuBisCo small subunit	O65194	5	25	206	20238	16000	8.86	8.3
R5	Glyceraldehyde 3 phosphate dehydrogenase	CAA36396	4	14	294	43312	38000	8.8	7.3
R6	Glyceraldehyde 3 phosphate dehydrogenase	AAA33780	4	11	98	43312	38000	8.8	7.9
R7	Unnamed protein product	CAA30978	5	18	147	40169	37000	8.56	7.2
R8	Malate dehydrogenase precursor (*Medicago sativa*)	AAB99755	5	18	261	35832	38000	8.8	6.6
R9	RuBisCo large subunit	AAD11774	5	11	129	50324	50,000	6.46	6.6
U1	RuBisCo activase (*Medicago sativa*)	AAN15964	12	47	631	29999	39000	5.63	5.7
U2	RuBisCo activase (*Medicago sativa*)	AAN15964	10	38	440	29999	39000	5.63	5.9
U3	ATP synthase beta subunit	AAD46914	22	47	865	52714	51000	5.14	5.6
U4	Heat Shock protein hsp 70	1909352A	6	9	266	75407	73000	5.22	5.2

[z]Spot ID is an arbitrary designation based on physical location of a protein spot on a gel used during protein identification.

major or minor modification of a specific protein on a plant's stability will need to be evaluated for the possibility of negative epistasic or plietropic effects. Finally, in vivo evaluation of modified alfalfa will be needed to confirm that ruminal degradation characteristics and the metabolizable protein supply to the ruminant animal have been improved.

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